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(57) Abstract

The invention relates to xylose isomerase mutants which express improved activity under acid pH conditions and/or increased thermal stability. Additionally, the affinity for metal ions may also be increased.

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XYLOSE ISOMERASE MUTANTS

This invention relates to mutants of the enzyme xylose isomerase which is an industrial enzyme used in the industrial conversion of glucose syrups obtained from corn or other starches or by hydrolysis of cellulose to sweeter sugars. Its effect is to convert glucose to high fructose syrups or xylose to xylulose, depending on the source material. This use has resulted in it being commonly called "glucose isomerase". Herein the correct nomenclature "xylose isomerase" will be used and, for the avoidance of doubt this term means those enzymes classed in Enzyme Classification EC 5.3.1.5, that is D-xylose ketol-isomerase. A review of the industrial uses of xylose isomerase appears in the reference Wen-Pin Chen, "Process Biochemistry" August/September 1980, pages 36 to 41 and C. Burke in Microbial Enzymes and Biotechnology, edit. W.M. Fogarty, Applied Science Publishers, pages 93 to 129, 1983.

More specifically the invention relates to xylose isomerase mutants which have improved activity under acidic pH conditions and/or increased thermal stability (with resulting increase in the enzyme life times when used at current processing temperatures of from 60 to 65^{o}C) and/ or affinity for metal ions.

Xylose isomerase occurs in numerous bacterial species but, although the enzyme action is similar throughout, there are variations in the composition of the protein and the complex molecular structure of the various forms of the enzyme. For example, in some variants the protein chains are longer than in others, some have dimeric protein chains and others are tetrameric. All known structures have the same general spatial conformation which includes a so-called "barrel" region formed by twisting of eight peptide chains in space about a longitudinal axis forming a cylinder of parallel beta-sheet (see K. Henrick et. al., Protein Engineering, 6, pages 467 to 469, 1987). The sites responsible for the enzyme action of the molecule are located at the carboxyl terminal end of the beta-strands which form the barrel. There is a high degree of conservation of the amino acid sequences for these sites amongst the enzymes from widely different bacterial sources, as would be expected from the near identity of their enzyme action. Most of the variations of the amino acid sequence that do occur, as a generalisation, are observed in regions of the molecule which do not participate in the enzyme action and which are remote from the enzymically active sites. As will be reported hereinafter, sequence and structure data confirm that high degree of conservation in the active regions with variations occurring elsewhere in themolecule.

One major industrial use of xylose isomerase is in the food industry for conversion of glucose to the sweeter fructose in the form of "high-fructose-containing-syrups", which has a market for caloric sweeteners used in solution , principally carbonated beverages.

Generally the enzyme is derived from the bacterial species of Streptomyces, Bacillus, Lactobacillus, Ampullariella and or Arthrobacter. However, the conversion of glucose to fructose is generally only one step of a multi-stage process from a raw material to the final sugar-containing product and other enzymes which have quite different functions are often used in sequential fashion. It is often the case that the process conditions, for example pH and temperature, under which optimum enzyme action is obtained, differ amongst the various enzymes used. These specific requirements of the naturally occurring enzymes are disadvantageous to the optimisation of the industrial processes which use them by requiring alteration of process parameters between the stages of the process.

A further problem in the industrial use of natural xylose isomerases is the fact that this enzyme is dependent on the presence of magnesium ion (and also cobalt(II) ion in some species) as an activator. All the natural xylose isomerases are inhibited by calcium ion which is present in starch feedstocks.

Calcium ions are required for the enzymic activity of alpha- amylase, which is used at about 100°C, pH 6 to 7, to convert starch to dextrose syrups in a batch process. The second step, also in batch, converts the resulting soluble dextrins to glucose, catalysed by the enzyme glucoamylase at 60°C and pH 5.5. The final glucose isomerisation is generally carried out at 60°C and pH 7 to 8 on columns of immobilised enzyme because the xylose isomerase is relatively costly and can thereby be used continuously for extended periods. Adjustments of pH and temperature and of the Ca^{2+}/Mg^{2+} balance are therfore necessary for the current technology. Ideally deionisation steps should

be left until all the enzyme steps have been completed, however, in the current industrial environment, calcium ion is necessarily removed upstream of the xylose isomerase step.

A further disadvantage of the conventional xylose isomerase step is the pH and temperature at which it is performed. If catalysed to equilibrium a glucose syrup would yield a mixture of 46.5% glucose and 53.5% fructose at 60°C but 41.4% glucose and 58.6% fructose at 80°C (Y. Takasaki, Agric. Biol. Chem., 31, page 309, 1980). Since fructose is much sweeter than glucose, the latter product would be more desirable. However, the constraints for an 80°C process of the xylose isomerases in current use are that undesirable "browning" reactions occur when sugars are heated at alkaline pH. Finally, the operational pH for xylose isomerase is incompatible with other enzymes used in the industrial processes, such as glucoamylase.

An object of the present invention is to obviate or mitigate the aforesaid disadvantages.

According to the present invention there is provided a xylose isomerase mutant in which at least one of the following mutations (Table I) in the naturally occurring protein sequence of the xylose isomerase, using the residue numbering system hereinafter defined, has been made:

(TABLE I)

Residue Number	Substituent
43	Cys
61	Arg, Lys, Gln or Asn
81	Cys
140	Arg, Lys, Gln or Asn
146	Cys

(TABLE I continued)

Residue Number	Substituent
171	Arg
180	Asp, Gln or Asn
189	Arg, Lys, Gln or Asn
199	Cys
200	Cys
203	Asp
204	Arg
216	Asp or Gln
220	Arg, Lys, Gln or Asn
223	Cys
244	Asn or Glu
253	Cys
254	Asn
256	Asn
263	Arg, Lys, Gln or Asn
292	Asn or Glu
355	Glu
380	Lys
370	Arg, Lys, Gln or Asn
382	Cys

Preferably the xylose isomerase is that derived from Arthobacter or from Streptomyces, Bacillus, Lactobacillus, Ampullariella and or Arthrobacter.

Therefore, according to the invention there is provided an Arthrobacter B3728 xylose isomerase, having one or more of the following (Table II) mutations:

(TABLE II)

Residue Number	Natural Residue	Substituent	Function
43	Ala	Cys	(b_1)
61	Asp	Arg, Lys, Gln or Asn	(a)
81	Thr	Cys	(b_1)
140	Glu	Arg, Lys, Gln or Asn	(a)
146	Gly	Cys	(b_1)
171	Lys	Arg	(b_2)
180	Glu	Asp, Gln or Asn	(c)
189	Asp	Arg, Lys, Gln or Asn	(a)
199	Leu	Cys	(b_1)
200	Ala	Cys	(b_1)
203	Glu	Asp	(b_2)
204	Gln	Arg	(b_2)
216	Glu	Asp or Gln	(c)

(TABLE II continued)

Residue Number	Natural Residue	Substituent	Function
220	Glu	Arg, Lys, Gln or Asn	(a)
223	Ala	Cys	(b_1)
244	Asp	Asn or Glu	(c)
253	Tyr	Cys	(b_1)
254	Asp	Asn	(c)
256	Asp	Asn	(c)
263	Asp	Arg, Lys, Gln or Asn	(a)
292	Asp	Asn or Glu	(c)
355	Ala	Glu	(b_2)
370	Glu	Arg, Lys, Gln or Asn	(a)
380	Ile	Lys	(b_2)
382	Leu	Cys	(b_1)

The functions of the mutations, indicated in Table II above, are as follows:

(a) Shifts the optimum pH of the enzyme activity towards a more acidic pH.

Figure 1 shows the structure of the active site bound Mg^{2+} and the inhibitor sorbitol (sorbitol is an acyclic analogue of the open chain structure of glucose which is the chemical configuration upon which the enzyme acts). It is probable that protonation of His219 is responsible for the reduction of activity at lower pH. It has been demonstrated in the case of the protease subtilisin that elimination of a negative charge lying 15 Angstroms away from the catalytic site reduces the pKa of the active site histidine in this enzyme by 0.6 pH units. The mutations of this invention which have this effect lie relatively close to the active site but in regions that appear to be unconnected with activity or stability. Hence a combination of these mutations will increase the activity at acid pH to the levels necessary for the target process. Those mutations where a non-essential negative charge is replaced by a positive charge (

residues 220,140,189,61,263 and 370 to be mutated to Lys or Arg) will have a greater effect than replacement of a negative charge by a neutral one (as in the subtilisin studies where Asp was changed to Ser), although mutations of the residues 220,140,189,263 and 370 in addition to Asn and Gln are also part of this invention.

(b) Improves the thermal stability of the enzyme

We have discovered that the pathway of denaturation of the protein by high temperature or by action of urea is to depolymerise to identical monomeric chains. In tetrameric configuration this reaction proceeds first to a dimeric structure (Figure 2) and then to four identical monomers. The first step occurs between 4M and 7M urea at 22°C with complete retention of activity in the dimers.

Figure 3 shows the specific activity of enzyme (0.7 mg/ml) incubated for one hour at 30°C in 10mM $MgCl_2$ - 50mM tris Cl, pH8 containing zero to 8M urea. Glucose isomerase assays were conducted on 20 microlitre samples at 30°C and pH8 in the presence (o) or absence (o) of urea at the relevant concentration. The bars show % of monomer (inactive) or dimer-tetramer equilibrium mixture (fully active) calculated from Fig. 2b below.

Figure 4 shows the elution profiles of the above samples separated at 22°C on DHEAE-Sephacel columns (9.5 cm x 0.7 cm diameter) equilibrated with urea-buffers of the same composition as the samples and a gradient from zero to 0.5M sodium chloride. So as to compensate for variability in column packing, the profiles are presented as absorbance versus increase in

effluent conductivity. The shift in peak position reflects rapid reversible equilibrium between tetramer at zero urea and predominantly dimer at 8M urea. A peak of inactive monomer is seen in the 5M and 8M urea incubations. The broken lines indicate the profiles of 8M urea samples after three days and then a further seven days incubation at 22°C showing the irreversible conversion of dimer to monomer.

Figure 5 shows tracings of "gradient-urea-" run (top to bottom) at 40 mV for 14 hours at pH8.5. The top gel is stained for total protein and the bottom gel for both protein and xylose isomerase activity. The change in mobility between 2M and 5M is consistent with a rapid reversibly equilibrium between tetramers and dimers that retain activity.

Hence it is clear that dissociation into active dimers is the first step in the pathway of inactivation. So the residues in contact at the subunit interface must be reponsible for stability. In Table II the function represented by (b_1) indicates those mutations which allow introduction of disulphide bonds at the various subunit interfaces.

In Table II, the function indicated by (b_2) is the introduction of salt bridges at the subunit interface surfaces (residues 203, 204, 171, 355 and 380) which results in increased thermal stability.

It is desirable to select more than one of the proposed mutations and the following are examples of particularly preferred selections.

(1) Ala43 to Cys43 and Thr81 to Cys81 to form a disulphide bridge between subunits (in A chain in Arthrobacter).

(2) Leu199 to Cys199 and Ala200 to Cys200 to form interchain disulphide bonds (between A and A' chains in Arthrobacter).

(3) Gly146 to Cys146 and Leu382 to Cys382 to form interchain disulphide bonds (between A and A' chains in Arthrobacter).

(4) Glu203 to Asp203 and Gln204 to Arg 204 to form a salt bridge at a subunit interface (between the A and A' chains in Arthrobacter).

(5) Tyr253 to Cys253 and Ala223 to Cys223 to form two disulphide bridges (between the A and B' and between the A and A' chains in Arthrobacter)

(6) Leu199 to Cys199, Ala200 to Cys200, Glu203 to Asp203 and Gln204 to Arg204 to introduce both disulphide and salt bridges within a short section of the peptide sequence.

(c) Reduces Calcium Ion Inhibition

X-ray diffraction data show that the sugar binds in the open chain form for the isomerisation step (Figure 6). Computer modelling and energy minimisation experiments indicate that neither a pyranose nor furanose ring can be bound to the enzyme at the isomerisation site., hence ring opening must precede isomerisation. The rate of ring opening which occurs in the absence of the enzyme is slow and the life-time and concentration of the open chain form in free solution is too low to supply the enzyme with a substrate in a form for the isomerisation to take place.

X-ray crystallographic studies of the binding of Mg^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+} and Pb^{2+} ions reveal two cation binding sites. One is the "isomerisation site" shown in Figure 1 which approximates to an octahedral arrangement of the protein side chains of Glu180, Glu216, Asp242 and Asp292. The second is close to it but is not involved in the isomerisation mechanism. For this site the coordinating groups of the protein, His219, Asp254, Asp256 and Glu216, give rise to an irregular arrangement of donor groups. It is possible to model pyranose and furanose rings at this "ring opening site" to provide a plausible mechanism for catalysis of ring opening by the bound Mg^{2+} (Figure 6). We have shown that calcium ions bind to this site with stronger affinity than magnesium ions. The greater versatility of Ca^{2+} over Mg^{2+} makes it ideally suited to be complexed by irregularly shaped protein cavities, together with the marked favouritism of complex ligand systems containing carboxylates for Ca^{2+} over Mg^{2+} would explain why Ca^{2+} inhibits the enzyme.

Mutations which reduce the binding affinity of Ca^{2+} at the "ring-opening site"are indicated by (c) in the "Function" column of Table II.

The amino acid residue numbering sequence used herein is based on the sequence of xylose isomerase derived from Arthrobacter strain B3728 and is shown in Fig. 7 herewith. The complete amino acid sequence of this particular enzyme is shown along with complete or partial sequences of xylose isomerases from other bacterial sources. The sequences of the various enzymes have been aligned with the Arthrobacter B3728

enzyme sequence by aligning the highly conserved regions of the sequences in which the desired mutations are most likely to be made. This diagram may be used to identify the locations in the sequences of enzymes from sources other than the Arthrobacter species for which at least a partial sequence, which includes some of the conserved regions, corresponding to the rim of the barrel region of the enzyme structure, is known. By aligning the conserved sequences and thereafter referring to the numbering of the Arthrobacter sequence, the appropriate locations for making the mutations defined above may be identified. The residues identified in Table III below are known to be conserved thoughout the xylose isomerase group of enzymes. The residue numbering refers to that of the xylose isomerase of Arthrobacter B3728.

TABLE III

Residue Number	Amino Acid
15	Trp
23	Asp
25	Phe
26	Gly
30	Arg
43	Ala
52	Phe
53	His
56	Asp
59	Pro
89	Thr
91	Asn
93	Phe
96	Pro
101	Gly
104	Thr
114	Ala
129	Gly
134	Val

TABLE III continued

Residue Number	Amino Acid
136	Trp
137	Gly
138	Gly
139	Arg
140	Glu
141	Gly
143	Glu
149	Asp
180	Glu
181	Pro
182	Lys
183	Pro
185	Glu
186	Pro
201	Phe
214	Asn
216	Glu
219	His
227	Phe
244	Asp
246	Asn
247	Gly
254	Asp
256	Asp
259	Phe
287	Gly
291	Phe
292	Asp
294	Lys
297	Arg
302	Asp
318	Leu
319	Lys

Referring now to Figure 7, the conserved residues are shown enclosed within boxes drawn in broken lines and it is these conserved residues which provide a key to the determination of the location of residues of species other than Arthrobacter which require to be mutated in accordance with this invention. The full or partial sequences of the xylose isomerases from the following sources are quoted.

Bs	=	Bacillus subtilis
Ec	=	Escherichia coli
Sg	=	Streptomyces griseofuscus
Sa	=	Streptomyces albus
Svr	=	Streptomyces violaceusruber
Svn	=	Streptomyces violaceoniger
Amp	=	Ampullariella sp Strain 3876
A	=	Arthrobacter Strain 3728

(The N-terminal methionine is not included in the numbering of the residues).

Further according to the present invention there is provided the nucleotide sequence of the XylA gene of Arthrobacter strain B3728, shown in Figure 8 herewith.

The invention also provides the nucleotide sequence shown in Figure 8 having one or more mutations at locations necessary to achieve one or more of the mutations listed in Table I above in the peptide expressed by the gene.

In addition, the invention provides an oligonucleotide comprising a fragment of the sequence shown in Figure 8 and containing at least one mutation.

The present invention also provides a host/vector expression system containing one of the mutant genes of this invention. The host may be E. coli or, more preferably, a yeast, preferably Saccharomyces cerevisiae.

Cloning, Site-directed Mutagenesis and Expression of Mutant Enzymes

The conventional strategy for cloning xylose isomerase from Arthrobacter would be to determine a partial amino acid sequence of the purified enzyme, construction of redundant oligo probes and the use of

radioactiovely labelled probes to screen a gene library consisting of a partial Sau 3A digest of Arthrobacter ligated into the Bam Hl site of pBR322. However, this conventional strategy failed to yield the gene of interest: instead it gave a gene which showed some sequence homology to the xylose isomerase gene but was much smaller.

Hence a different strategy was used. By selecting mutants of Arthrobacter which were unable to grow on xylose/minimal medium (after muatgenesis with N-methyl-N'-nitro-N- nitrosoguanidine and mutant enrichment on glucose/minimal medium containing 20 micrograms/ml of ampicillin). One such mutant, strain PCl, contained a lesion in its gene for xylose isomerase but not in its xylulokinase (Xyl B) gene or in its inducibility by xylose as shown by the enzyme activities in crude extracts shown in Table IV below.

TABLE IV

XI (XI) Xylulokinase (XK)
Levels in B3724 and PCl

Strain	Induced	Specific Activity XI (10^{-3})	XK (10^{1-})
B3724	-	2.0	N.D.
	+	128.0	7
PCl	-	0.2	N.D.
	+	0.6	22

Cells were grown overnight at 30°C in 40ml M9 medium with 0.2% casein hydrolysate. Enzymes were induced by the inclusion of 0.2% of D-xylose. Cell-free extracts were prepared according to Smith (1980). Protein concentration in the extracts was determined by the Bio-Rad (Trade Mark) assay kit. Levels of xylose isomerase and xylulokinase were determined as described by Smith (q.v.) and Briggs (1983) respectively. Specific Activity of xylose isomerase and xylulokinase are defined as micromoles of D-xylose isomerised per milligram of protein per minute at 20°C and as micromoles of D-xylulose phosphorylated per milligram of protein per minute at 20°C. (N.D. = not detected)

We have developed novel vectors that allow transformation of Arthrobacter strains B3724 and PC1 at high frequencies, adequate for screening of gene banks by complementation [P-C Shaw et.al, J. Gen. Microbiol. 134, 903-911 (1988)]. Gene banks of B3728 DNA were constructed by ligating 5-10 Kb fragments cleaved to completion with ClaI, SalI or Bam HI sites in pCG2100. The ligation mixture was transformed to protoplasts of strain PC1 and regenerated in agar in the presence of kanamycin and sodium succinate as osmotic stabiliser. Clones containing a functional Xyl A gene in the regenerated colonies were detected by their ability to grow on minimal agar plates with xylose as the carbon source. Eight such colonies from a SalI gene bank contained identical 4.8 kilobase inserts. Deletions in this insert followed by subclonings and retransformations located the Xyl A gene in a 1.9 kilobase SalI-BssHII fragment, whose sequence is shown in Figure 8. It encodes the amino acid sequence also shown in Figure 8. The plasmid is named pAXI1.

Site directed mutagenesis can be carried out on this fragment by standard methods in M13 phage (Zoller, M. J. and Smith M. Methods Enzymol. 100, 468 - 500, 1983) or by ligating it into the Sma I site in plasmid pTZ19U (D.A. Mead, E. Szczensna-Skopura and B. Kemper, Protein Engineering, 1, 67 -74, 1986). This allows hybridisation with oligonucleotide primers to be carried out directly on the single strand form of the plasmid, according to the method of these authors. The primers are designed to produce codon changes resulting in the amino acid replacements shown in Table I. As example, the following mutant genes have been constructed via the synthetic oligonucleotides listed, and the expected codon changes have been confirmed by DNA sequencing of the plasmid:-

1) Residues 203 and 204, Glu - Asp and Gln - Arg
Oligonucleotide: GCC.TTC.ATC.GAC.CGT.CTG.GAG.CAC.
.(GAG) (CAG)
Mutagenesis technique: pTZ19U

2) Residue 140, Glu = Lys
Oligonucleotide: C.GGG.CGC.AAG.GGC.AGC.G
(GAA)
Mutagenesis technique: M13

3) Residue 136, Trp = Glu
Oligonucleotide: TTC.GTC.ATG.GAA.GGC.CCC.C
(TGG)
Mutagenesis technique: M13

4) Residue 189, Asp = Lys
Oligonucleotide: A.CGC.GGC.AAG.ATC.TTC.C
(GAC)
Mutagenesis technique: M13

Expression of wild-type or mutant XylA genes can conveniently be studied directly in the E.coli host JA221 since it contains a mutation in the E.coli XylA gene. Hence growth on xylose depends on the expression of the foreign gene. Somewhat surprisingly E.coli appears to recognise the Arthrobacter XylA promoter, and since the plasmid is multicopy, levels of Arthrobacter xylose isomerase are similar to that in the original Arthrobacter B3728 (Table V).

TABLE V

Host	Plasmid	Medium: Glucose minimal	Xylose minimal	G +X minimal	LB rich
Ar. B3724	a)pAXI1	0.258	1.464	0.440	0.050
(XI inducible)	b)none	0.008	1.1000	0.019	0.025
	a)-b)	0.250	0.364	0.421	0.025
Ar. B3728	a)pAX1	1.470	1.300	-	0.720
(XI constitutive)	b)none	1.030	1.000	1.090	0.650
	a)-b)	0.440	0.400	-	0.70
Ar. PC1	a)pAXI1	0.050	0.560	0.390	0.040
(XI^-, XK	b)none	0.002	N.G.	0.020	0.0044
inducible)	a)-b)	0.048	(0.560)	0.370	0.036
EC JA221	a)pAXI1	0.150	0.380	0.200	0.076
(XI^-, XK	b)none	0.000	N.G.	0.000	0.000
inducible)	a)-b)	0.150	(0.380)	0.200	0.076
EC JM101	a)pAXI2	1.000	-	-	0.190
(XI inducible)	b)none	0.090	-	-	0.060
(IPTG induced)	a)-b)	0,910	-	-	0.130

All activities are in micromoles fructose per minute per microgram of protein in cell extractsa, determined by the cysteine-carbazole assay of Z. Dische and E. Borenfreund (J. Biol. Chem., 192, 583-587, 1951). Strains of Arthrobacter are derived from strain

B3724 as described above. E. coli strain JA221 is recA1 leuB6 trp ΔE5 $hsdM^+$ $hsdR^-$ lacY xyl C600 (Briggs et.al. EMBO J., 3, 611-616, 1984) and E.coli strain JM101 is Δ lacpro supE thiF' tra D36 pro AB $lacI^q$ Z Δ M15 (Messing et.al., Proc. Natl. Acad. Sci. USA, 74, 3642-3646, 1977). pAXI2 contains the sequence shown in Fig 8 ligated into site pTZ19U. N.G. indicates no growth.

However, it is not particularly envisaged that the enzyme for industrial use would be produced in E.coli, since this would be doubtfully food-compatible. It is preferable that the mutant enzyme be substituted in Arthrobacter Strain B3728 for the natural Xyl A and used in an immobilised enzyme process. This is achieved by a technique of 'gene disruption' using the shuttle vector pCG2100 which has been constructed for this purpose.

However, the properties of the improved enzyme of this invention are such that it is admirably suited to a system in which it is expressed in yeast. Yeast is of course food compatible and there exist many vectors for Saccharomyces cerevisiae that allow high expression of foreign proteins. Since the yeast can be produced inexpensively and since its functions will be destroyed by the high-temperature (80°C) of the target progress, the cells will have xylose isomerase activity as their sole enzymic function. Hence the use of an S.cerevisiae expressing one or other of the improved xylose isomerases listed in Table 1 in a batch process or in a continuous immobilised cell process is an important embodiment of this invention.

CLAIMS

1. A Xylose isomerase mutant with improved activity having at least one of the following mutations in the naturally occuring protein sequence of the xylose isomerase:-

Residue Number	Natural Residue	Substituent
43	Ala	Cys
61	Asp	Arg, Lys, Gln or Asn
81	Thr	Cys
140	Glu	Arg, Lys, Gln or Asn
146	Gly	Cys
171	Lys	Arg
180	Glu	Asp, Gln or Asn
189	Asp	Arg, Lys, Gln or Asn
199	Leu	Cys
200	Ala	Cys
203	Glu	Asp
204	Gln	Arg
216	Glu	Asp or Gln
220	Glu	Arg, Lys, Gln or Asn
223	Ala	Cys
244	Asp	Asn or Glu
253	Tyr	Cys
254	Asp	Asn
256	Asp	Asn
263	Asp	Arg, Lys, Gln or Asn
292	Asp	Asn or Glu
355	Ala	Glu
370	Glu	Arg, Lys, Gln or Asn
380	Ile	Lys
382	Leu	Cys

2. A mutant according to Claim 1 characterised in that the isomerase is preferably derived from Arthobacter, Streptomyces, Bacillus, Lactobacillus, Ampullariella, or Arthrobacter,

3. A mutant according to any of claims 1 or claims 2 characterised in that it is derived from Arthobacter B3728 xylose isomerase having at least one of the following mutations:-

Residue Number	Natural Residue	Substituent
43	Ala	Cys
61	Asp	Arg, Lys, Gln or Asn
81	Thr	Cys
140	Glu	Arg, Lys, Gln or Asn
146	Gly	Cys
171	Lys	Arg
180	Glu	Asp, Gln or Asn
189	Asp	Arg, Lys, Gln or Asn
199	Leu	Cys
200	Ala	Cys
203	Glu	Asp
204	Gln	Arg
216	Glu	Asp or Gln
220	Glu	Arg, Lys, Gln or Asn
223	Ala	Cys
244	Asp	Asn or Glu
253	Tyr	Cys
254	Asp	Asn
256	Asp	Asn
263	Asp	Arg, Lys, Gln or Asn
292	Asp	Asn or Glu
355	Ala	Glu
370	Glu	Arg, Lys, Gln or Asn
380	Ile	Lys
382	Leu	Cys

4. A mutant according to any of the preceding claims characterised in that it displays an enzyme activity towards a more acid pH having residues 220, 140, 189, 263, and 370 mutated to lysin or arginin.

5. A mutant according to claims 1 to 3 characterised in that it displays an enzyme activity towards a more acid pH having residues 220, 140, 189, 263, and 370 mutated to Asn and Gln.

6. A mutant according to any of the preceding claims characterised in that it displays improved thermal stability of the enzyme by introduction of salt bridges at residues 203, 204, 171, 355 and 380.

7. A mutant according to any of the preceding claims characterised in that the thermal stability of the enzyme is achieved by mutations:-

(1) Ala43 to Cys43 and Thr81 to Cys81 to form a disulphide bridge between subunits (in A chain in Arthrobacter).

(2) Leu199 to Cys199 and Ala200 to Cys200 to form interchain disulphide bonds (between A and A' chains in Arthrobacter).

(3) Gly146 to Cys146 and Leu382 to Cys382 to form interchain disulphide bonds (between A and A' chains in Arthrobacter).

(4) Glu203 to Asp203 and Gln204 to Arg 204 to form a salt bridge at a subunit interface (between the A and A' chains in Arthrobacter).

(5) Tyr253 to Cys253 and Ala223 to Cys223 to form two disulphide bridges (between the A and B' and between the A and A' chains in Arthrobacter)

(6) Leu199 to Cys199, Ala200 to Cys200, Glu203 to Asp203 and Gln204 to Arg204 to introduce both disulphide and salt bridges within a short section of the peptide sequence.

8. A mutant according to any of the preceding claims characterised in that it shows reduced calcium ion inhibition if residue 180 is mutated to Asp, Gln, or Asn, 216 to Asp or Gln, 244 to Asn or Glu, 254 to Asn, 256 to Asn and 292 to Asn or Glu.

9. A mutant according to any of the preceding claims 1 - 8 characterised in that it is contained in a host.

10. A mutant according to claim 9 characterised in that the host is E. coli or S. cerevisiae.

11. A mutant according to any of the preceding claims characterised in that conservative residues in xylose isomerases of different origin are mutated in accordance with the mutations described.

12. A xylose isomerase characterised by the nucleotide sequence of the XylA gene of Arthrobacter strain B3728 and mutations thereof.

13. An oligonucleotide for a xylose isomerase sequence fragment having at least one mutation therein.

14. A vector characterised in that it allows strains Arthrobacter and PC1 to be transformed at high frequencies.

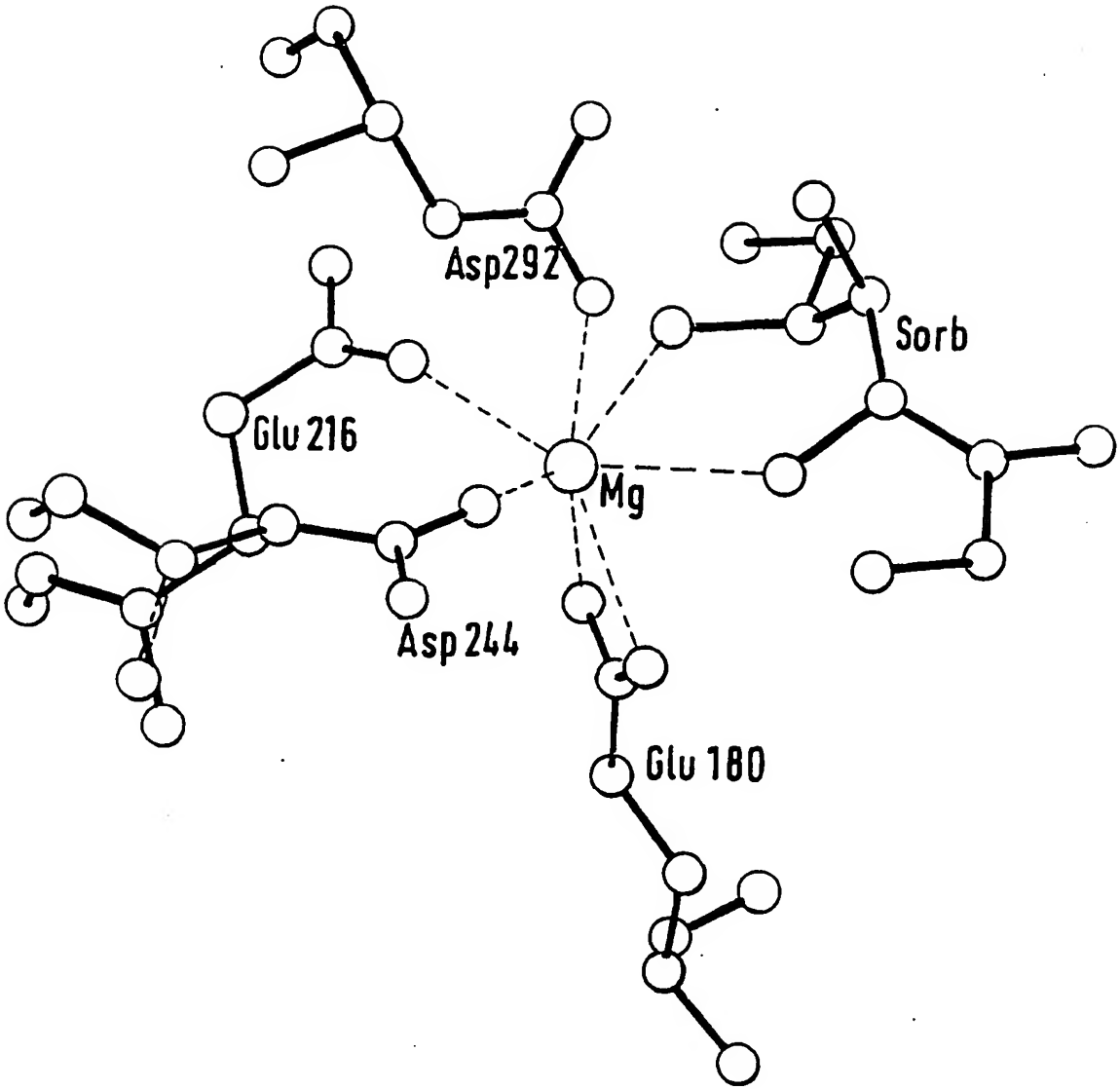
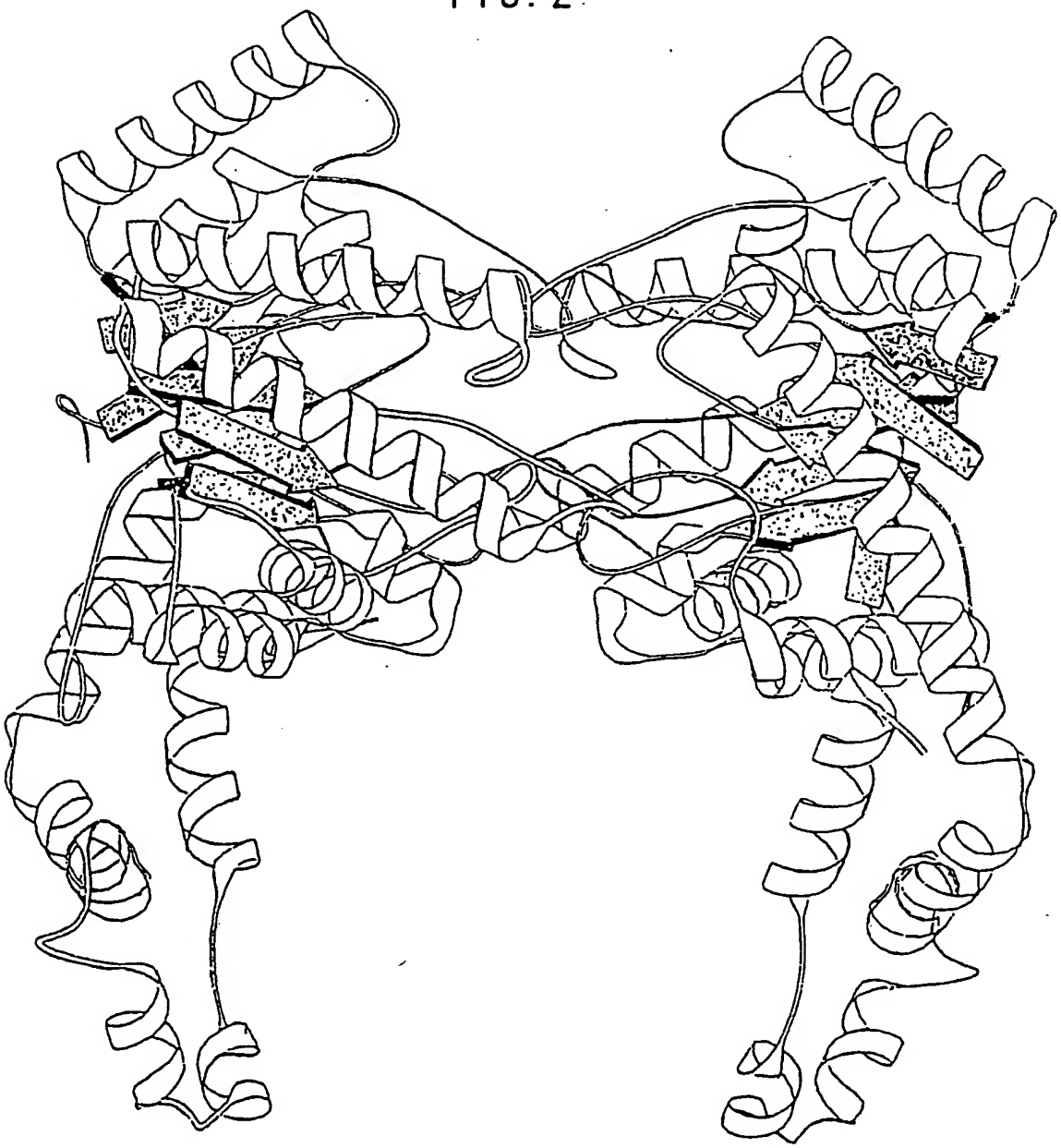


FIG.1

FIG. 2



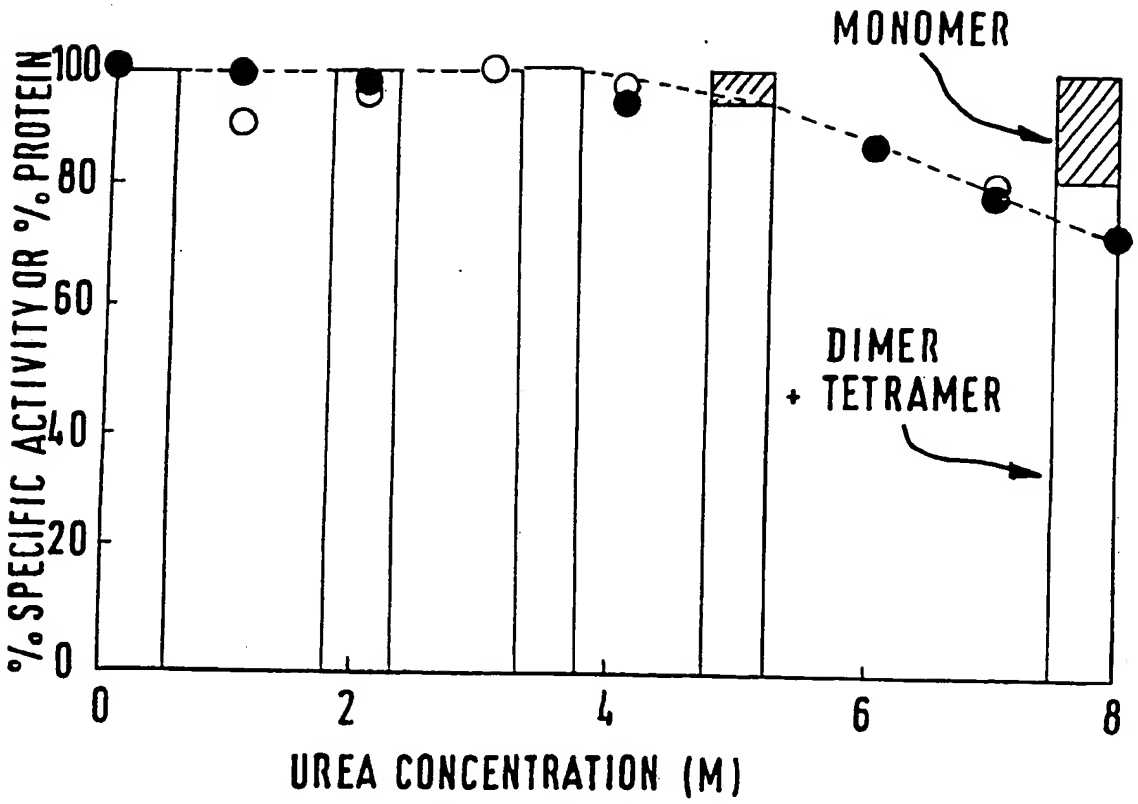

MONOMER
DIMER
+ TETRAMER
% SPECIFIC ACTIVITY OR % PROTEIN
100
80
60
40
20
0
0
2
4
6
8
UREA CONCENTRATION (M)

FIG. 3

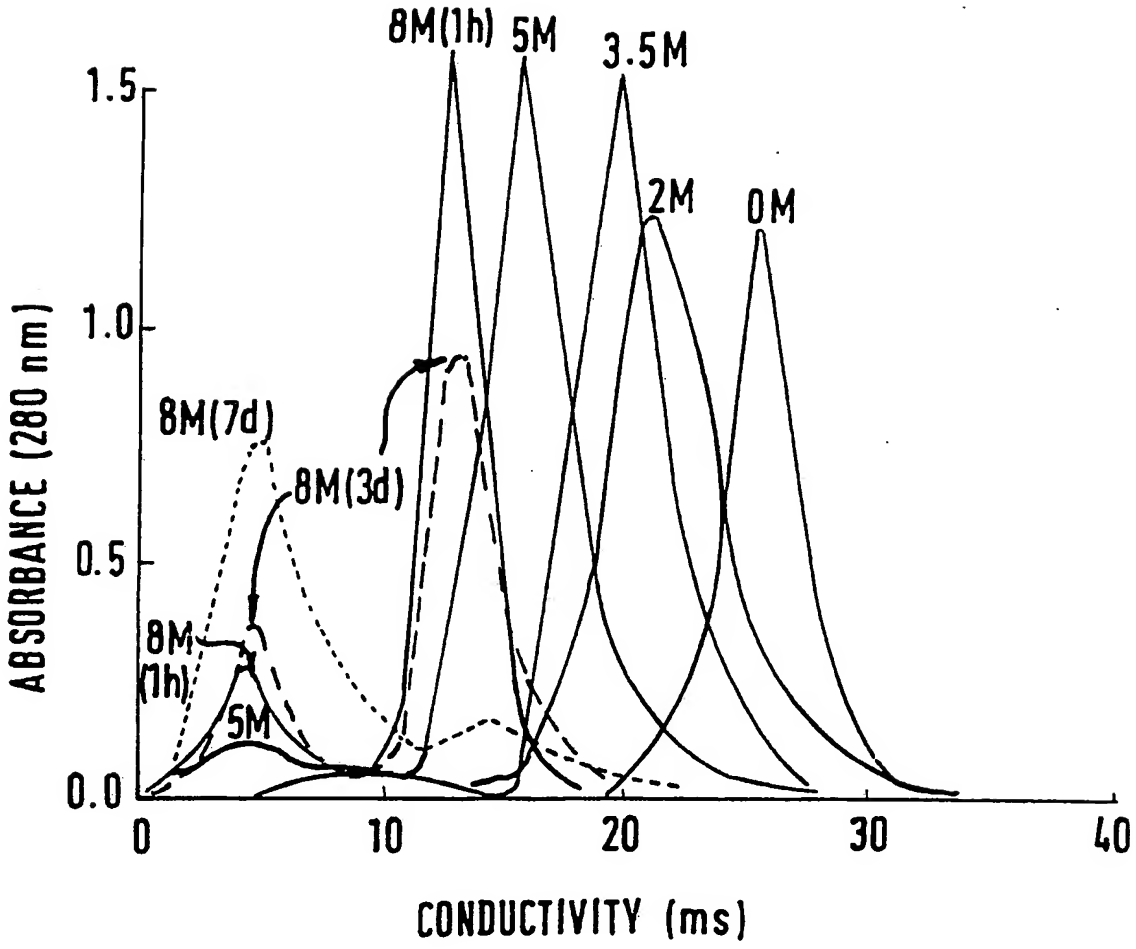
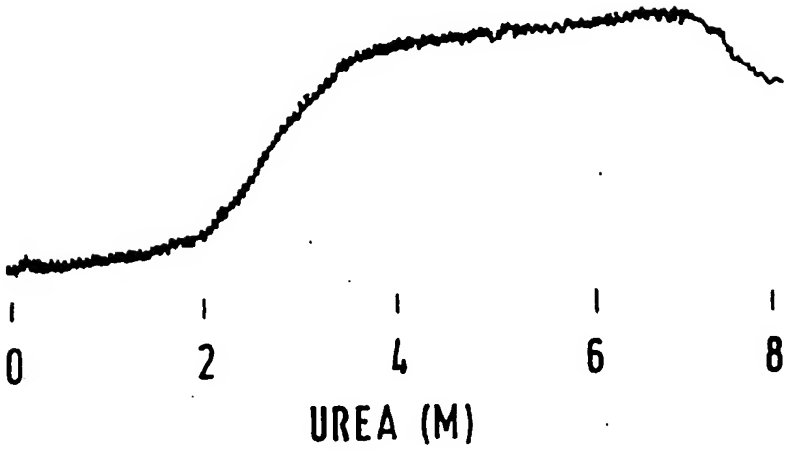

8M(1h)
5M
3.5M
2M
0M
8M(7d)
8M(3d)
8M (1h)
5M
1.5
1.0
0.5
0.0
ABSORBANCE (280 nm)
0
10
20
30
40
CONDUCTIVITY (ms)

FIG. 4

i) PROTEIN STAIN



ii) ACTIVITY STAIN

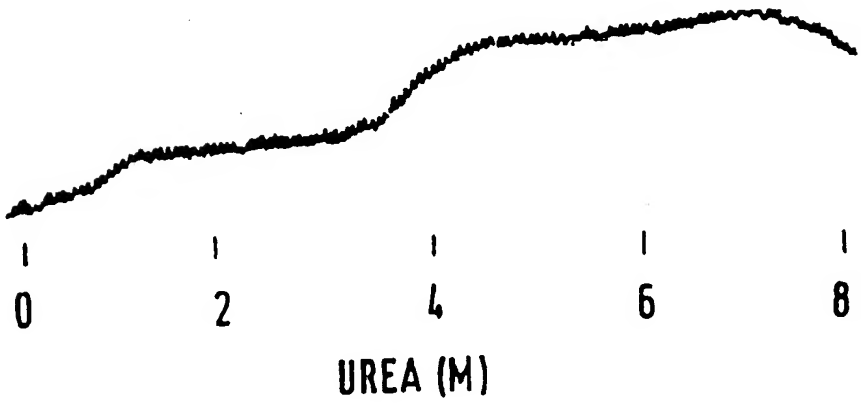


FIG. 5

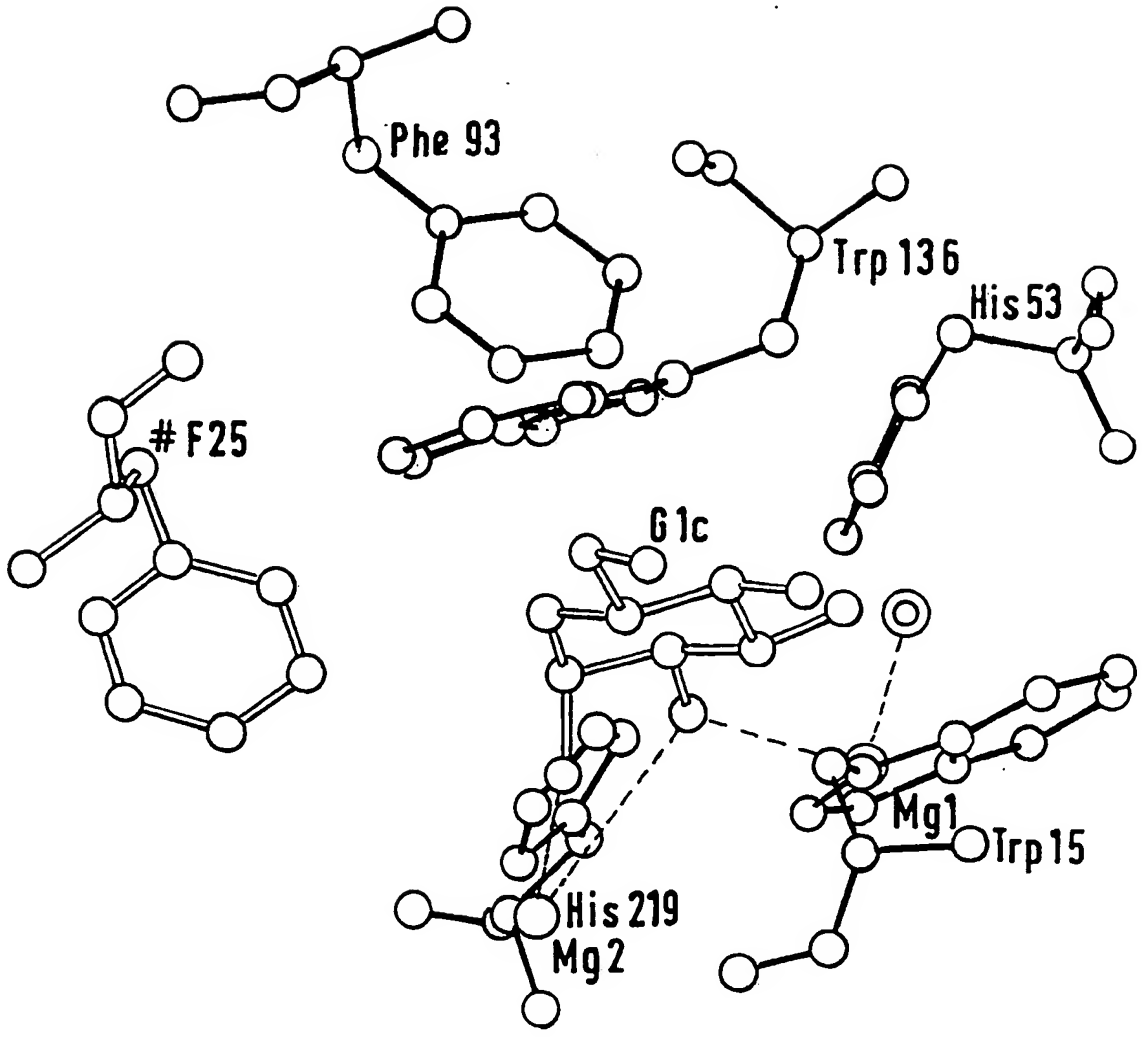


FIG.6

```
  1 MAQSHSSSVNYFGSVNKVVFEGKASTNPLAFKYYNPQEVIGGKTMKEHLRFSIAYWHTFTADGTDV  65 Bs
  1        MQAYFDQLDRVRYEGSKSSNPLAFRHYNPDELVLGKRMEEHLRFAACYWHTFCWNGADM  58 Ec
  1                                          MSDQ                        3 Sg
  1                                          MNYQP                       4 Sa
  1                                          MNYQPTPEDRFTF               12 Svr
  1                                          MSFQPTPEDKFTFGLWTVG WQGRDP  24 Svn
  1                                          MSLQATPDDKFSFGLWTVG WQARDA  24 Amp
  1                                          MSVQPTPADHFTFGLWTVG WTGADP  24 Λ
                                                bbbbbhhhh

 66 FGAATMQRPWDHYKGMDLARARV  EAAFEMFEKLDAPFFAFIHNRDIAPEGSTLKETNQNLDIIVG 129 Bs
 59 FGVGAFNRPWQQP GEALALAKRKADVAFEFFHKLHVPFYCFHDVDVSPEGASLKEYINNFAQMVD 123 Ec
 25 FGDAT  RPALDPVETVQRLAEL           GAYGVTFHDDDLVPFGSS    DTERESHIK  72 Svn
 25 FGDAT  RPVLDPIEAVHKLAEI           GAYGVIFHDDDLVPFGAD    AATRDGIVA  72 Amp
 25 FGVAT  RKNLDPVEAVHKLAEL           GAYGITFHDNDLIPFDAT    EAEREKILG  72 Λ
              hhhhhhhhhhh              bbbb  hhhhh          hhhhhhhhh

130 MIKDYMRDSNVKLLWNTANMFTNPRFVHGAATSCNADVFAYAAAQVKKGLETAKELGAENYVFWGG 195 Bs
124 VLAGKQEESGVKLLWGTANCFTNPRYGAGAATNPDPEVFSWAATQVVTAMEATHKLGGENYVLWGG 189 Ec
 73 RFRQALDATGMTVPMATTNLFTHPVFKDG FTANDRDVRRYALRKTIRNIDLAAELGAK..... 137 Svn
 73 GFSKALDETGLIVPMVTTNLFTHPVFKDGGFTSNDRSVRRYAIRKVLRQMDLGAELGAKTLVLWGG 138 Amp
 73 DFNQALKDTGLKVPMVTTNLFSHPVFKDGGFTSNDRSIRRFALAKVLHNIDLAAEMGAETFVMWGG 138 Λ
    hhhhhhhhh  bbbbb                hhhhhhhhhhhhhhhhhhhhh   bbbb
```

FIG.7

```
196 REGYETLLNTDLKFELDNLARFMHMAVDYAKEIEYTGQFLIEPKPKEPTTHQYDTDAATTIAFLKQ 261 Bs
190 REGYETLLNTDLRQEREQLGRFMQMVVEHKHKIGFQGTLLIEPKPQEPTKHQYDYDAATVYGFLKQ 255 Ec
138 ...I.I.....DVRDALDRMKEAFDLLGEYVTAQGYDLRFAIEPKPNEPRGDILLPTVGHALAFIER 203 Svn
139 REGAEYDSAKDVGAALDRYREALNLLAQYSEDQGYGLPFAIEPKPNEPRGDILLPTAGHAIAFVQE 204 Amp
139 REGSEYDGSKDLAAALDRMREGVDTAAGYIKDKGYNLRIALEPKPNEPRGDIFLPTVGHGLAFIEQ 204 A
                 hhhhhhhhhhhhhhhhhhhhh     bbbbb                hhhhhhhhhh

262 YGLDNHFKLNLDANHATLAGHTFEHELRMARVHGLLGSVDANQGHPLLGWDTDE FPTDLYSTTLA 326 Bs
256 FGLEKEIKLNIEANHATLAGHSFHHEIATAIALGLFGSVDANRGDAQLGWDTDQ FPNSVEENALV 320 Ec
204 LERPELYGVNPEVGHEQMAGLNFPHGIAQALWAGKLFHIDLN GQSGIKYDQDLRFGAGDLRAAFW 268 Svn
205 LERPELFGINPETGHEQMSNLNFTQGIAQALWHKKLFHIDLN GQHGPKFDQDLVFGHGDLLNAFS 269 Amp
205 LEHGDIVGLNPETGHEQMAGLNFTHGIAQALWAEKLFHIDLN GQRGIKYDQDLVFGHGDLTSAFF 269 A
       bbbbbbbhhhhhhhhh  hhhhhhhhhh  bbbbbbb                  hhhhhh

327 MYEILQNGGL      GSGGLNFDAKVRRSSFEPDDLVYAHIAGMDAFARGLKVAHKLIEDRVFED 386 Bs
321 MYEILKAGGF      TTGGLNFDAKVRRQSTDKYDLFYGHIGAMDTMALALKIAARMIEDGELDK 380 Ec
269 LVDLLESAGY        GPRHFDFKPPRTED  FDGVWASAEGCMRNYLILKERAAAFRANPEVQ 324 Svn
270 LVDLLEN G PDGGPAYDGPRHFDYKPSRTED  FDGVWESAKDNIRMYLLLKERAKAFRADPEVQ 331 Amp
270 TVDLLEN GFPNGGPKYTGPRHFDYKPSRTDG  YDGVWDSAKANMSMYLLLKERALAFRADPEVQ 332 A
    hhhhhhh            bbbb          hhhhhhhhhhhhhhhhhhhhhhhhhhhh hhhh
```

FIG. 7 (continued)

```
387 VIQHRYRSFTEGIGLEITEGRANFHTLEQYAL  NNKTIKNESGRQERLKPILNQ          439 Bs
381 RIAQRYSGWNSELGQQILKGQMSLADLAKYAQ EHHLSPVHQSGRQEQLENLVNHYLFDK   439 Ec
325 EALRAARLNQLAQPTAAD GLEALLADRTAFE DFDVEAAAARAAWPFERLDQLAMDHLLGARG 386 Svn
332 AALAESKVDELRTPTLNPGETYADLLADRSAFEDYDADAVGAKG YGFVKLNQLAIDHLLGAR  393 Amp
333 EAMKTSGVFELGETTLNAGESAADLMNDSASFAGFDAEAAAERN FAFIRLNQLAIEHLLGSR  394 A
    hhhhhh hhhhh      hhhhhh      hhhhhh      hhhhhhhhhhhhhh
```

FIG.7 (continued)

FIGURE 8

Sequence of the Xyl A gene from Arthrobacter B3728

```
  1  CGT CGA CCG CGT TCC CCG TCG CGG TGC ACG CCG ATT TCG ATC ACT AGC   48
 49  CCG GCG GCC AGC AGG TCG GTG ACC AGA CTA GAG ACC GAG GCC TTG GTG   96
 97  AGC TGG CTG AGT TGG GCG ATA TCG GCG CGT GAT AAC CGC TGG TCA TCT  144
145  CCC GCT GCG GCA ATC ACC GAC AGC ACC CTG GAC AGG TTG GCT TTG CGC  192
193  ACG TCC CCG ACG TTG CCC GGG GCC GAG GAT GTT GCG GCT CGG GCG GTT  240
241  GTC GCT GGT TGG CGC ATG CCT TCT CCT TGT GGA AAT TTC TTG AAT GGA  288
289  TTC GTA GGG CTC GCT ATT GAC TCT AGC GCA TCA TCA CCC ATA TAG TTC  336
337  AGG ACA TAA ACT AAA TGG CAT CAG CCA ACC CCG ACG ATC CAA GGA TGT  384

385  ATC TCA ATG AGC GTT CAG CCG ACC CCT GCA GAC CAC TTC ACC TTT GGC  432
           MET/Ser Val Gln Pro Thr Pro Ala Asp His Phe Thr Phe Gly

433  CTC TGG ACC GTA GGA TGG ACC GGC GCC GAC CCA TTC GGT GTC GCC ACC  480
     Leu Trp Thr Val Gly Trp Thr Gly Ala Asp Pro Phe Gly Val Ala Thr

481  CGC AAG AAC CTG GAC CCG GTA GAA GCC GTC CAC AAG CTG GCC GAG CTC  528
     Arg Lys Asn Leu Asp Pro Val Glu Ala Val His Lys Leu Ala Glu Leu

529  GGC GCC TAC GGC ATC ACC TTC CAC GAC AAT GAC CTG ATT CCT TTT GAC  576
     Gly Ala Tyr Gly Ile Thr Phe His Asp Asn Asp Leu Ile Pro Phe Asp
```

Figure 8 (continued)

```
577  GCC ACC GAG GCA GAG CGC GAA AAG ATC CTT GGT GAC TTC AAC CAG GCG  624
     Ala Thr Glu Ala Glu Arg Glu Lys Ile Leu Gly Asp Phe Asn Gln Ala

625  CTG AAG GAC ACC GGC CTG AAG GTC CCA ATG GTG ACC ACC AAC CTG TTC  672
     Leu Lys Asp Thr Gly Leu Lys Val Pro MET Val Thr Thr Asn Leu Phe

673  AGC CAC CCG GTC TTC AAG GAC GGC GGC TTC ACC TCT AAC GAC CGC TCG  720
     Ser His Pro Val Phe Lys Asp Gly Gly Phe Thr Ser Asn Asp Arg Ser

721  ATC CGT CGT TTT GCA CTG GCT AAG GTC CTG CAC AAC ATC GAC TTG GCA  768
     Ile Arg Arg Phe Ala Leu Ala Lys Val Leu His Asn Ile Asp Leu Ala

769  GCC GAG ATG GGC GCC GAA ACC TTC GTC ATG TGG GGC GGG CGC GAA GGC  816
     Ala Glu MET Gly Ala Glu Thr Phe Val MET Trp Gly Gly Arg Glu Gly

817  AGC GAA TAC GAC GGT TCC AAG GAC CTG GCC GCA GCA CTT GAT CGC ATG  864
     Ser Glu Tyr Asp Gly Ser Lys Asp Leu Ala Ala Ala Leu Asp Arg MET

865  CGC GAA GGC GTG GAC ACG GCA GCT GGC TAC ATC AAG GAC AAG GGT TAC  912
     Arg Glu Gly Val Asp Thr Ala Ala Gly Tyr Ile Lys Asp Lys Gly Tyr

913  AAC CTG CGC ATC GCG CTG GAG CCA AAG CCA AAT GAA CCA CGC GGC GAC  960
     Asn Leu Arg Ile Ala Leu Glu Pro Lys Pro Asn Glu Pro Arg Gly Asp
```

Figure 8 (continued)

```
961   ATC TTC CTG CCT ACC GTC GGC CAC GGC CTG GCC TTC ATC GAG CAG CTG
      Ile Phe Leu Pro Thr Val Gly His Gly Leu Ala Phe Ile Glu Gln Leu
                                                                  1008

1009  GAG CAC GGC GAC ATC GTC GGC CTG AAC CCA GAA ACC GGC CAC GAG CAG
      Glu His Gly Asp Ile Val Gly Leu Asn Pro Glu Thr Gly His Glu Gln
                                                                  1056

1057  ATG GCC GGC CTG AAC TTC ACC CAC GGC ATC GCT CAG GCA CTG TGG GCC
      MET Ala Gly Leu Asn Phe Thr His Gly Ile Ala Gln Ala Leu Trp Ala
                                                                  1104

1105  GAG AAG CTG TTC CAC ATT GAC CTC AAC GGC CAG CGC GGC ATC AAG TAC
      Glu Lys Leu Phe His Ile Asp Leu Asn Gly Gln Arg Gly Ile Lys Tyr
                                                                  1152

1153  GAC CAG GAC CTG GTC TTC GGC CAC GGC GAT CTG ACC AGC GCG TTC TTC
      Asp Gln Asp Leu Val Phe Gly His Gly Asp Leu Thr Ser Ala Phe Phe
                                                                  1200

1201  ACC GTA GAC CTG CTG GAA AAC GGC TTC CCT AAC GGC GGA CCA AAG TAC
      Thr Val Asp Leu Leu Glu Asn Gly Phe Pro Asn Gly Gly Pro Lys Tyr
                                                                  1248

1249  ACC GGC CCA CGC CAC TTC GAC TAC AAG CCA TCG CGC ACC GAC GGC TAC
      Thr Gly Pro Arg His Phe Asp Tyr Lys Pro Ser Arg Thr Asp Gly Tyr
                                                                  1296

1297  GAC GGC GTG TGG GAC TCG GCC AAG GCC AAC ATG TCC ATG TAC CTG CTG
      Asp Gly Val Trp Asp Ser Ala Lys Ala Asn MET Ser MET Tyr Leu Leu
                                                                  1344
```

Figure 8 (continued)

```
1345  CTC AAG GAA CGT GCC CTG GCC TTC CGT GCG GAT CCA GAG GTA CAG GAA  1392
      Leu Lys Glu Arg Ala Leu Ala Phe Arg Ala Asp Pro Glu Val Bln Glu

1393  GCC ATG AAG ACC TCG GGC GTC TTC GAA CTG GGC GAA ACC ACC CTG AAC  1440
      Ala MET Lys Thr Ser Gly Val Phe Glu Leu Gly Glu Thr Thr Leu Asn

1441  GCC GGG GAA AGC GCA GCG GAT CTG ATG AAT GAT TCC GCG AGC TTC GCA  1488
      Ala Gly Glu Ser Ala Ala Asp Leu MET Asn Asp Ser Ala Ser Phe Ala

1489  GGC TTT GAC GCC GAG GCC GCC GCA GAG CGC AAC TTC GCG TTC ATC CGC  1536
      Gly Phe Asp Ala Glu Ala Ala Ala Glu Arg Asn Phe Ala Phe Ile Arg

1537  CTG AAC CAG CTG GCC ATC GAG CAC CTG CTC GGC TCC CGC TAA ACC CTG  1584
      Leu Asn Gln Leu Ala Ile Glu His Leu Leu Gly Ser Arg  *

1585  TCT GAA CCC ACC GTA GAA AGC AGC CAC ATT CAA TGA CGC TTG TAG CCG  1632
1633  GCA TCG ACT CCT CCA CCC AGT CTT GCA AAG TTG TCA TCC GTG ACG CCG  1680
1681  ATA CCG GAG TGC TCA TCC GCT CCT CAC GTG CCA GTC ACC CGG ATG GCA  1728
1729  CGG AAG TAG ACC CGG AGT TCT GGT TCG ATG CCT TGC AAG AAG CGA TCG  1776
1777  CCC AGG CCG GAG GCC TGG ATG ATG TGG CTG CGA TCT CGG TGG GCG GGC  1824
1825  AGC AGC ACG GCA TGG TGG CGC TAG ATT GGC CAC CCG GTG CGG TGA TCC  1872
1873  GCC CTG CGC TGC TGT GGA ATG ACA ACC GCA GCG CGC                  1908
```

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 89/00748

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC 5: C 12 N 9/92, C 12 N 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC 5	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Protein Engineering, volume 1, no. 3, April 1987, P.-C. Shaw et al.: "Protein engineering of Arthrobacter glucose isomerase", page 264 see abstract no 154 --	1-5,9-11
P,Y	WO, A, 89/01520 (CETUS CORPORATION) 23 February 1989 see claims --	1-5,9-11
A	US, A, 4410627 (LLOYD et al.) 18 October 1983 see column 3, lines 11-65; claims 1,5,6	

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* Special categories of cited documents: [10]

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"P" document published prior to the international filing date but later than the priority date claimed

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22nd September 1989	13. 11. 89
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	T.K. WILLIS

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 8900748 SA 29852

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The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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US-A- 4410627	18-10-83	AU-B- 558696	05-02-87
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		CA-A- 1200521	11-02-86
		DE-A- 3323617	19-01-84
		FR-A,B 2529572	06-01-84
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		SE-A- 8303725	31-12-83